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On the Mechanism of the Reaction of Tris(hydroxymethyl)aminomethane with Activated Carbonyl Compounds: A Model for the Serine Proteinases

JOHN DE JERSEY, ANN K. FIHELLY, AND BURT ZERNER

Department of Biochemistry, University of Queensland, St. Lucia, Queensland, Australia 4067

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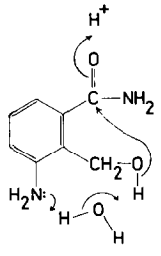
The pH dependence of the reaction of tris(hydroxymethyl)aminomethane (Tris) with the activated carbonyl compound 4-*trans*-benzylidene-2-phenyloxazolin-5-one (I) is given by the equation $k_2' = k_b K_a/(K_a + [H^+]) + k_a[OH^-]K_a/(K_a + [H^+])$, where K_a is the dissociation constant of $TrisH^+$. Spectrophotometric experiments show that the Tris *ester* of α-benzamido-*trans*-cinnamic acid is formed quantitatively over a range of pH values, regardless of the relative contribution of k_b and k_a terms to k_2'. Hence, both terms refer to *alcoholysis*. While the mechanism of the reaction is not determined unequivocally in the present work, the magnitude of the k_b term, together with its dependence on the basic form of Tris, suggests that ester formation is occurring by nucleophilic attack of a Tris hydroxyl group on the carbonyl carbon of the oxazolinone, with intramolecular catalysis by the Tris amino group. The rate enhancement due to this group is at least 10^2 and possibly of the order 10^6. This system is compared with other model systems for the acylation step of catalysis by serine esterases and proteinases.

INTRODUCTION

The acylation step in catalysis by serine-dependent esterases and proteinases involves general base catalysis by a histidine imidazole group of the attack of a serine hydroxyl group on the carbonyl carbon atom of the bond being cleaved (*1*). Data on model systems for this reaction which include a general base and a hydroxyl group in the same molecule are very limited. Fife and Benjamin (*2*) studied the cyclization of 3-amino-2-hydroxymethylbenzamide, showing that the presence of the adjacent amino group increases the pH-independent rate by a factor of 10^3. However, steric restrictions and the magnitude of $\Delta S\ddagger$ suggest that catalysis occurs through one or more water molecules as in Scheme I. A much



Scheme I

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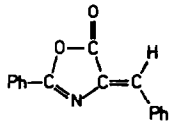
greater rate enhancement might be expected if the functional groups were aligned to permit direct removal of the hydroxyl proton by the amino group. Other model systems have either used water molecules to provide the hydroxyl group (*3*, *4*), or have otherwise separated the hydroxyl group and the general base (*5*, *6*).

In an earlier study (*7*) of the reaction of 4-*trans*-benzylidene-2-phenyloxazolin-5-one (I)[1] with tris(hydroxymethyl)aminomethane (Tris), spectral evidence was



I

obtained for the formation of a transient intermediate prior to the formation of the Tris amide of α-benzamido-*trans*-cinnamic acid. The evidence was consistent with the identification of the intermediate as the Tris ester of α-benzamido-*trans*-cinnamic acid, with possible catalysis of ester formation by the amino group of Tris. A similar mechanism has been proposed for the reaction of Tris with *p*-nitrophenyl acetate (*9*), based on the magnitude of the rate constant for *p*-nitrophenol release, and the reactivity of the first product with alkaline hydroxylamine. Subsequently, Bruice and York (*10*) reported on a detailed study of the reactions of Tris and pentaerythritol with a series of phenyl acetates. They showed that the apparent second-order rate constant (k_2') for the reaction with Tris is given by Eq. [1]:

$$k_2' = k_b K_a/(K_a + [H^+]) + k_a[OH^-]K_a/(K_a + [H^+]) \qquad [1]$$

where K_a is the dissociation constant of $TrisH^+$. For pentaerythritol, in which the amino group of Tris is replaced by a fourth $-CH_2OH$ group, Eq. [1] simplifies to Eq. [2]:

$$k_2' = k_a[OH^-]. \qquad [2]$$

Since k_a values for Tris and pentaerythritol are similar, the hydroxide-catalyzed reaction of Tris with phenyl acetates was taken to represent formation of the Tris ester in a reaction which is not catalyzed by the amino group. The value of k_b for Tris is not greatly different from that expected for a hindered amine acting as a nucleophile. Hence, the k_b term was taken to represent a simple aminolysis of the ester. Further evidence for this intrepretation was provided by considering the reaction of acetic anhydride with Tris. By potentiometric titration of acetate ions produced and amino groups remaining, the ratio of aminolysis to alcoholysis could be determined. It was found that under all conditions examined (pH 5–10), *aminolysis* was the predominant reaction.

Since all our previous studies (*7*) of the reaction of I with Tris were performed at a single pH (pH 8.0), the possibility remained that formation of the intermediate ester was simply due to the k_a step of Eq. [1]. In the present experiments, we have (i) shown that the pH dependence of the reaction of I with Tris is described by Eq.

[1] The isomer of I with mp 167°C used in this and the previous work (*7*) has been shown to be the *trans*-isomer (*8*).

[1]; (ii) shown that the intermediate ester is formed *quantitatively* in the pH range 8.12–9.59, thereby demonstrating that both k_b and k_a steps lead to ester formation; (iii) considered the reactions of I with pentaerythritol and glycine ethyl ester, allowing an estimate to be made of the rate enhancement due to the participation of the Tris amino group in the reaction.

EXPERIMENTAL

Materials. I was prepared as described previously (*7*). Tris (Trizma Base, Reagent Grade) and glycine ethyl ester hydrochloride were obtained from Sigma Chemical Co., and acetonitrile (Spectro Grade) and pentaerythritol from Eastman Organic Chemicals. Other buffer components were analytical grade reagents. Ionic strength was maintained constant using KCl. Measurements of pH were made on a Radiometer pH meter 4, standardized according to Bates (*11*), and are accurate to ±0.01 pH units unless otherwise specified. Care must be taken when using glycine ethyl ester–HCl buffers especially at high pH and concentration because of the relatively facile formation of dipeptide esters and diketopiperazines (*12*). Such buffers were prepared just before use from stock glycine ethyl ester hydrochloride. In all kinetic experiments, the pH of the reaction mixture was determined immediately after spectral observation was terminated.

Methods. All spectrophotometric measurements were made at 25°C using a Cary 17 or a Cary 14 recording spectrophotometer. The decrease in concentration of I was followed at 363 nm ($\Delta\epsilon = -36{,}000$). The formation and decay of the intermediate in the reaction of I with Tris was followed at 310 nm. The Cary 17 spectrophotometer was fitted with digital equipment allowing absorbance-time data to be collected on punch tape. First-order rate constants were calculated from such data using a computer program devised to permit evaluation by both infinity and Guggenheim methods. In all kinetic experiments, the concentration of the nucleophile was much greater than that of I, and clean first-order kinetics were observed.

The reaction of I with Tris was studied as follows. Tris–HCl buffer (3 ml; 0.01, 0.02, 0.05, 0.10, and 0.20 *M*; pH 7.01, 7.53, 7.99, 8.51, 9.01, 9.47; $\mu = 0.2$) and acetonitrile (250 μl) were equilibrated at 25 ± 0.1°C, and the reaction initiated by the addition of 50 μl of I in acetonitrile, giving $[S]_0 = 9.09 \times 10^{-7}$ *M*, [acetonitrile] = 9.1% v/v. Duplicate determinations were made in each buffer. The reaction of I with glycine ethyl ester was studied in the same way, using glycine ethyl ester–HCl buffers (0.01, 0.02, 0.05, 0.10, and 0.20 *M*; pH 6.9, 7.5, 8.0, 8.3, 8.5, and 8.7; $\mu = 0.2$). The reaction of I with pentaerythritol was observed in 0.05 *M* barbital buffer (pH 8.47, $\mu = 0.2$), and in 0.1 *M* carbonate buffer (pH 9.67, $\mu = 0.2$), with pentaerythritol concentration 0 – 0.27 *M* [acetonitrile] = 8.0% v/v. Data were evaluated using pK_w, 14.14; pK_a' $TrisH^+$, 8.14; pK_a' glycine ethyl ester–HCl, 7.80, the measured values in 9% v/v acetonitrile, $\mu = 0.2$, 25°C.

RESULTS

Reaction of I with Tris and Pentaerythritol

First-order rate constants (k_{obs}) were determined in five Tris–HCl buffers (9.1–

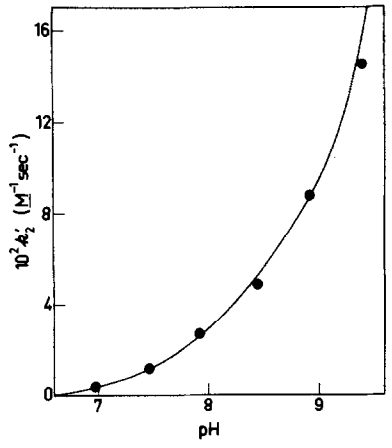


FIG. 1. pH-rate profile for the reaction of I with Tris: solid line calculated from Eq. [1].

182 mM) at each of six pH values from 6.97 to 9.40. In all cases, plots of k_{obs} against $[Tris]_{total}$ were linear, indicating the absence of a second-order term in [Tris] under these conditions, and gave values for k_2' at each pH. Inspection of the data suggested that Eq. [1] might apply, and a plot of k_2' $(K_a + [H^+])/K_a$ against $[OH^-]$ was linear within experimental error, yielding values of k_b (0.067 M^{-1} sec^{-1}) and k_a (5.4×10^3 M^{-2} sec^{-1}). Figure 1 shows a plot of k_2' against pH, the solid line being that calculated from Eq. [1] using these values of k_a and k_b. The intercepts of the plots of k_{obs} against $[Tris]_{total}$ were replotted against $[OH^-]$ to give a value of 130 M^{-1} sec^{-1} for k_{OH^-} in the hydrolysis of I.

Values of k_2' were similarly determined for the reaction of I with pentaerythritol from the linear plots of k_{obs} against pentaerythritol concentration at pH 8.47 and

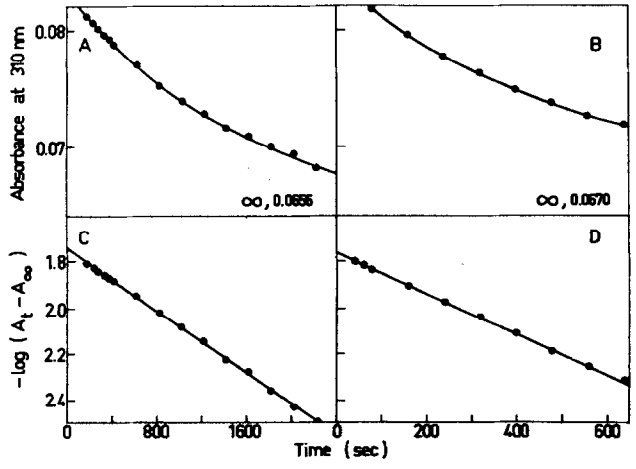


FIG. 2. Progress curves observed at 310 nm (A,B) and corresponding first-order plots (C,D) for the reaction of I in 1.0 M Tris–HCl buffers at pH 8.12 (A) and pH 9.59 (B). The solid lines in C and D were determined by least squares analysis.

TABLE 1

EFFECT OF pH ON INTERMEDIATE FORMATION IN THE REACTION OF I WITH TRIS AT 25°C[a]

pH	ΔA at 310 nm[b]	λ_{max}[c] (nm)	$10^2\ k_{obs}$ (363 nm) (sec^{-1})	$10^2\ k_{obs}$ (310 nm)[d] (sec^{-1})
8.12	0.0187 ± 0.0002	282	2.96	3.11
	0.0186 ± 0.0002			
8.58	0.0181 ± 0.0003	282	4.64	4.69
	0.0186 ± 0.0003			
9.11	0.0178 ± 0.0002	282	7.91	8.30
	0.0184 ± 0.0002			
9.59	0.0174 ± 0.0002	282	15.6	17.3
	0.0184 ± 0.0002			
	0.0171 ± 0.0002			

[a] The reaction mixtures contained 3 ml of 1.0*M* Tris–HCl buffer (μ = 0.5), 250 μl of acetonitrile, and 50 μl of I (5.52×10^{-4} *M* in acetonitrile).

[b] Repeat determinations.

[c] λ_{max} of final product.

[d] Values of k_{obs} were determined by back extrapolation of the initial rate of the slow reaction to zero time, and considering the ΔA between this extrapolated line and the measured absorbance at time t as $A_\infty - A_t$. Failure to allow exactly for the second reaction probably explains the slightly higher estimates of k_{obs} at 310 nm.

pH 9.67. These values were consistent with Eq. [2], yielding a k_a of $6.9 \pm 0.2 \times 10^3$ M^{-2} sec^{-1}. The product of the reaction of I with pentaerythritol has λ_{max} 287 nm (ϵ = 14,950).

When the reaction of I with Tris is observed at 310 nm, an initial rise in absorbance is followed by a much slower fall, indicating the formation and decay of an intermediate. The effect of pH on the amount of intermediate formed was determined by observing the reaction of I with Tris at 310 nm in 1.0 *M* Tris–HCl buffers, pH 8–9.5. Figure 2 shows typical progress curves, together with first-order plots corresponding to the slow reaction. By extrapolation of the first-order plots to zero time, values of ΔA for the slow reaction were obtained (Table 1). Product spectra at each pH showed λ_{max} 282 nm, corresponding to the Tris amide of α-benzamido-*trans*-cinnamic acid (*7*). Hence, ΔA is a measure of the amount of intermediate formed. Included in Table 1 are the apparent first-order rate constants for the loss of I, measured at 363 nm, and for the formation of the intermediate, measured at 310 nm in the same buffers.[2] The observed agreement between these rate constants is required by the proposition that the progress curves at 310 nm represent the formation of an intermediate from I and its subsequent decay.

[2] I undergoes additional reactions in aqueous solution which are not first-order in [I]. Under all conditions used in this work, clean first-order kinetics are observed. These additional reactions make it difficult to estimate k_{obs} for the reaction of I with H_2O, and can become significant in Tris buffers when [Tris] is low and [I] is high.

Reaction of I with Glycine Ethyl Ester

Plots of k_{obs} against [glycine ethyl ester]$_{total}$ showed upward curvature, slight at the low pH values and marked at the high pH values, indicating the presence in the rate equation of a second-order term in glycine ethyl ester concentration. Previous studies (*13*) have indicated that the reaction of glycine ethyl ester with an activated ester in aqueous solution is likely to be described by Eq. [3]:

$$k_{obs} = k_0 + k_2[GEE] + k_3[GEE]^2 + k_4[GEE][OH^-] \quad [3]$$

where $k_0 = k_{H_2O} + k_{OH^-}[OH^-]$, and GEE is the basic form of glycine ethyl ester. If so, a plot of $(k_{obs} - k_0)/[GEE]$ against [GEE] at any pH should be linear, with an intercept of $k_2 + k_4[OH^-]$, and a slope of k_3. Plots of the experimental data were linear, as was a replot of the intercepts against $[OH^-]$, giving the rate constants: $k_2 = 0.172\,M^{-1}\,sec^{-1}$; $k_3 = 1.4 \pm 0.2\,M^{-2}\,sec^{-1}$; $k_4 = 1.97 \times 10^4\,M^{-2}\,sec^{-1}$. Figure 3 shows plots of k_{obs} against [glycine ethyl ester]$_{total}$ at three pH values; the solid lines are calculated from Eq. [3] using the above values for the rate constants, with k_0 negligible.

DISCUSSION

The reactions of Tris (Fig. 1) and pentaerythritol with I follow the same rate equations (Eqs. [1] and [2]) as those established for the corresponding reactions with phenyl acetate (*10*). Values of k_a for Tris ($5.4 \times 10^3\ M^{-2}\ sec^{-1}$) and pentaerythritol ($6.9 \times 10^3\ M^{-2}\ sec^{-1}$) are very similar if allowance is made for the different numbers of hydroxyl groups. The spectrum of the product of the pentaerythritol reaction (λ_{max} 287 nm; ϵ = 14,950) is consistent with its

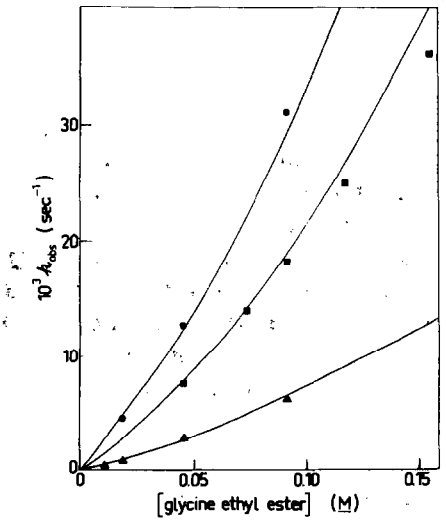


FIG. 3. Effect of pH on the reaction of I with glycine ethyl ester: ●, pH 8.71; ■, pH 8.23; ▲, pH 7.50; solid lines calculated from Eq. [3].

identification as the ester (cf. methyl α-benzamido-*trans*-cinnamate, λ_{max} 285 nm; ϵ = 15,830). Hence, the k_a term in the rate equations represents hydroxide-catalyzed alcoholysis of I, with no catalysis by the Tris amino group.

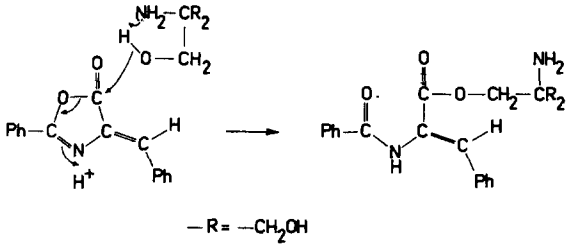
The main point at issue is the reaction represented by the k_b term of Eq. [1]: simple aminolysis to give the Tris amide *or* alcoholysis to give the Tris ester with intramolecular catalysis by the amino group. An intermediate can be observed spectrally in the reaction of Tris with I (Fig. 2) and the results presented in Table 1 show that in 1.0 *M* Tris–HCl buffers from pH 8.12 to 9.59, the same amount of intermediate is formed (assuming that the spectrum of the intermediate is independent of pH in this region). Since the reaction of Tris with I is described by Eq. [1], and since k_a and k_b are known, the contribution of the k_a term to k_2' can be calculated as ~7% at pH 8.12 and ~70% at pH 9.59. Therefore, if the k_b term represents aminolysis to give the stable Tris amide, the amount of intermediate formed at pH 9.59 would be 10 times that formed at pH 8.12. Since the k_a term leads to ester formation (cf. Tris and pentaerythritol), the k_b term must also give ester; otherwise, the amount of intermediate formed would change drastically with pH. Further, since both k_a and k_b terms lead to ester formation, and since these are the only terms in Eq. [1], which describes the reaction of Tris with I, ester formation must be quantitative. A number of further arguments support the identification of the intermediate as the Tris ester: (i) intermediate formed at pH 8, where the k_b term greatly predominates has λ_{max} ~286 nm and ϵ ~18,000, values very similar to those of both methyl and pentaerythritol esters of α-benzamido-*trans*-cinnamic acid; (ii) the isolated intermediate has earlier (*7*) been shown to be a substrate for α-chymotrypsin, with Tris as a hydrolysis product, whereas the Tris amide is not a substrate; (iii) the $\Delta\epsilon$ at 310 nm for conversion of intermediate (ester) to amide observed in the experiments of Table 1 (2,150) is also consistent with quantitative formation of ester in the k_2' step.[3]

The k_b term in Eq. [1] therefore represents alcoholysis. The dependence of k_b on the basic form of Tris (Eq. [1]), together with the magnitude of k_b (see below), suggest that the Tris amino group is participating in the reaction.

One possible mechanism for the k_b term is the nucleophilic attack on I by the zwitterion $^+H_3N–C(CH_2OH)_2–CH_2O^-$ ($Tris^{\pm}$). It is possible to calculate the zwitterionic ratio $(Tris^{\pm})/(Tris^0)$, if we assume a value for the microscopic dissociation constant for the reaction $TrisH^+ \rightleftarrows Tris^{\pm} + H^+$. Using choline ($pK_a'$ 13.9) as a model compound, and allowing for the three hydroxyl groups of Tris, $(Tris^{\pm})/(Tris^0) = 5 \times 10^{-6}$. Hence, since $k_b = 0.067\ M^{-1}\ sec^{-1}$, the second-order rate constant for the reaction of $Tris^{\pm}$ with I would be $0.067/5 \times 10^{-6}$, i.e., $1.3 \times 10^4\ M^{-1}\ sec^{-1}$. Comparison of this value with k_{OH^-} ($130\ M^{-1}\ sec^{-1}$) shows that the zwitterion would need to be ~100 times as reactive as OH^-, despite being a much weaker base, and despite the steric hindrance.

An alternative mechanism is intramolecular general base catalysis by the Tris amino group (Scheme 2).

[3] Compare methyl cinnamate (λ_{max} 279.5 nm, ϵ = 22,200) with cinnamamide (λ_{max} 276 nm, ϵ = 21,300). At 304 nm, 28 nm from the λ_{max} of the amide, the $\Delta\epsilon$ for the conversion of ester to amide is 2400 (B. Zerner, unpublished results).



Scheme 2

As shown in Fig. 3, the reaction of I with glycine ethyl ester is described by Eq. [3]. This rate expression is clearly consistent with the proposition that nucleophilic attack by the amino group of glycine ethyl ester on the carbonyl group of I is subject to general base catalysis by the amino group of a second molecule of glycine ethyl ester (k_3 step) or by OH^- (k_4 step). The existence of the k_3 step therefore comments favorably on the feasibility of Scheme 2.

The magnitude of k_b in the reaction of Tris with I indicates immediately that intramolecular catalysis by the amino group is occurring. Thus, k_b (0.067 M^{-1} sec^{-1}) is not much smaller than k_2 for the aminolysis of I by glycine ethyl ester (0.172 M^{-1} sec^{-1}). It is difficult to estimate the rate of the uncatlayzed reaction of a Tris hydroxyl group with I in aqueous solution.[2] Indeed, it is difficult to measure the rate constant for the bimolecular reaction of any neutral hydroxyl group with a carbonyl group in aqueous solution because, as shown here for the reaction of I with pentaerythritol, and elsewhere (*14*), Eq. [2] applies under all accessible conditions. Considering our data on the reaction of I with pentaerythritol at pH 8.47, the rate constant for the reaction of neutral alcohol with I (k_b) must be negligible compared with k_a [OH^-]. This sets an upper limit of $\sim 7 \times 10^{-4}$ M^{-1} sec^{-1} for k_b, making the rate enhancement due to the Tris amino group ≥ 100. Another approach is to estimate k_b for the uncatalyzed reaction of a Tris hydroxyl group with I. In the absence of additional data, this may be achieved by assuming $k_{\text{Tris hydroxyl}} \doteq k_{H_2O\text{ hydroxyl}}$, estimating k_{H_2O} from the ratio of the k_2 values for the reaction of glycine ethyl ester and water with *p*-nitrophenyl acetate. This ratio is 7×10^6 in favor of aminolysis over hydrolysis (*9*). Such a calculation leads to 2.5×10^{-8} M^{-1} sec^{-1} (i.e., $0.172/7 \times 10^6$) as an estimate of k_b for the uncatalyzed reaction of a Tris hydroxyl group with I. To the extent that the assumptions used are approximately valid, they lead to an estimate of the order $\sim 10^6$ for the rate enhancement of alcoholysis due to the Tris amino group.

It seems likely that the reaction of Tris with other carbonyl compounds proceeds according to Scheme 2, but few data are available. For example, *trans*-cinnamoylimidazole reacts with Tris at pH 9.2 to form the ester rather than the amide (*15*). However, demonstration of the participation of the amino group in ester formation would require a similar kinetic analysis to that described in this paper. Similarly, the present work indicates that *p*-nitrophenyl acetate probably reacts with Tris according to Scheme 2. However, the present work was made possible by the spectral properties of I and its derivatives, and similar direct

observation of intermediates in the *p*-nitrophenyl acetate system is clearly not possible.

It may be argued that the present model for the acylation step is an incomplete one because of the purported involvement in that step of an aspartic carboxyl group in the "charge relay system" (*16*). The most convincing evidence for the involvement of an aspartic carboxyl group is the presence of such a group within hydrogen bonding distance of the active site histidine imidazole group in both the chymotrypsin family and the subtilisin family of serine proteinases (*16, 17*). These families of enzymes appear to have developed similar catalytic mechanisms by means of convergent evolution. The contribution, if any, of this aspartic carboxyl group to the overall rate enhancement is unknown but the following points should be noted:

(i) a small contribution to the overall catalysis (a factor of ≤ 10) might be sufficient to explain the "ubiquitous" presence of this group adjacent to the active site histidine group;

(ii) in model systems developed to mimic the proposed charge relay system (*3, 4*), the additional enhancement by the adjacent carboxylate ion of general base catalysis of ester hydrolysis by the imidazole group is small (<10);

(iii) as discussed by Bruice (*1*), the inversion of the pK_a' values of the imidazolium and carboxyl groups inferred from nmr studies of α-lytic protease (*18*) would not be expected to result in enhanced catalysis.

Therefore, it remains important to determine what proportion of the rate enhancement shown by the serine proteinases can be achieved in model systems such as the one described in this work.

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